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(71) Applicant: LOMA LINDA UNIVERSITY [US/US]; School of Medicine, Dept. of Microbiology & Molecular Genetics, Loma Linda, CA 92350 (US).

(72) Inventors: LANGRIDGE, William, H., R.; 11856 Westminster Court, Loma Linda, CA 92354 (US). ARAKAWA, Takeshi; 1–29–15, Tsuboya, Naha, Okinawa 902 (JP). CHONG, Daniel; Apartment #161, 2618 Flint Way, San Bernardino, CA 92408–3800 (US). MERRITT, John, Laurence; 1400 Barton Road 1916, Redlands, CA 92373 (US).

(74) Agents: MEADOWS, James, H. et al.; McDermott, Will & Emery, Suite 300, 99 Canal Center Plaza, Alexandria, VA 22314 (US).

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(54) Title: EXPRESSION OF CHOLERA TOXIN B SUBUNIT IN TRANSGENIC PLANTS AND EFFICACY THEREOF IN ORAL VACCINES

(57) Abstract

A gene encoding the cholera toxin B subunit protein (CTB) is inserted into a plant expression vector. A preferred embodiment has the CTB gene fused to an endoplasmic reticulum (ER) retention signal (SEKDEL) adjacent to the mannopine synthase P2 promoter and has a bacterial luciferase reporter gene (*lux F*) linked to a P1 promoter. Potato leaf explants transformed by *Agrobacterium tumefaciens* carrying the vector and kanamycin–resistant plants are regenerated. The plant–derived CTB is antigenically indistinguishable from bacterial CTB, and oligomeric CTB molecules ($M_r \sim 50kDa$) are the dominant molecular species isolated from transgenic potato leaf and tuber tissues. The maximum amount of CTB detected in auxin–induced transgenic potato leaf and tuber tissues is approximately 0.3 % of total soluble plant protein. Both serum and intestinal CTB–specific antibodies were induced in orally immunized mice. Mucosal antibody titers declined gradually after the last immunization but were restored following an oral booster of transgenic potato. The expression of oligomeric CTB with immunological and biochemical properties identical to native CTB in edible plants permits inexpensive food plant–based oral vaccines for protection against cholera and other pathogens in endemic areas throughout the world.

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EXPRESSION OF CHOLERA TOXIN B SUBUNIT IN TRANSGENIC PLANTS AND EFFICACY THEREOF IN ORAL VACCINES

Reference to Related Application

The present application is related to U.S. provisional application 60/061,265, filed October 7, 1997.

Technical Field

The present invention is related to genetic engineering of plants. The invention is particularly related to the production of transgenic plants that express immunogenic proteins, which are referred to herein as "edible vaccines."

Background of the Invention

Cholera is a devastating, infectious diarrheal disease that has caused recurrent pandemics throughout the world. Effective prevention of cholera dissemination depends on safe water sources and improved sanitation in countries where cholera is endemic. The poverty in these countries makes the cost of building large-scale water treatment and sanitation systems prohibitive. Thus, the ultimate challenge for cholera prevention lies in establishment of low-cost alternatives, including effective oral vaccines, which provide lasting protection after a single immunization, or convenient and readily available vaccines for frequent administration to people living in regions where cholera is endemic. It is widely recognized that oral vaccination is more effective than parenteral vaccination against pathogens that invade through mucosal surfaces. Moreover, oral vaccines are easier and safer to administer.

Based on the fact that cholera is controlled more effectively through mucosal rather than parenteral immunization, both killed whole-cell and recombinant live-attenuated oral cholera vaccine candidates have been extensively tested in field trials (Holmgren, J. *et al.*, 1996; Finkelstein, R., 1995; Mekalanos, J., 1994; Waldor, M., 1996). The oral administration of *Vibrio cholerae* enterotoxin (CTX) and its nontoxic B subunit (CTB) induce both systemic and mucosal antibody production in animals and man (Fujita

and Finkelstein, 1972; Fuhrman and Cebra, 1981; Lycke *et al.*, 1983; Elson and Ealding, 1984; Lycke *et al.*, 1985). CTX also acts as a strong immunological adjuvant for co-administered antigens (Lycke and Holmgren, 1986; Jackson *et al.*, 1993; Holmgren *et al.*, 1993). The strong adjuvant effect of CTX is due to its ability to interact with many vertebrate cell types by elevating intracellular cAMP levels through activation of adenylate cyclase (Gill and Meren, 1978).

Although, CTB is not generally considered to be a strong adjuvant for co-administered antigens, it has been demonstrated to be an effective carrier molecule for induction of mucosal immunity to polypeptides to which it is chemically or genetically conjugated (McKenzie and Halsey, 1984; Czerkinsky *et al.*, 1989; Dertzbaugh and Elson, 1993). In addition, CTB has been shown to function as a carrier of conjugated peptides for induction of immunological tolerance (Sun *et al.*, 1994, 1996). The characteristic of CTB as an immunomodulatory carrier molecule may be due largely to its ability to avidly bind to its natural receptor G_{M1}-ganglioside, on the surface of mammalian intestinal epithelial cells, including M cells of the gut-associated lymphoid tissues (GALT) (Svennerholm, 1976).

The development of transformation methods for stable integration of foreign DNA into plants (Schell, 1987) has enabled the production of transgenic plants capable of expressing a wide variety of foreign genes, e.g., bacterial luciferase in tobacco (Koncz *et al.*, 1987), secretory antibodies in tobacco (Ma *et al.*, 1995), and the human milk protein β-casein in potato (Chong *et al.*, 1997). Additionally, microbial plant-based vaccines have been used proposed for oral immunization in mammals (Haq *et al.*, 1995; Thanavala *et al.*, 1995; Mason *et al.*, 1996).

Previously, it is unknown, however, whether CTB subunits can be produced in transgenic food plant tissues for assembly into the pentameric structure, which is essential for high binding affinity for the natural toxin receptor (G_{M1}-ganglioside). It is also unknown if the subunits can stimulate a significant protective immune response against the biological effects of cholera holotoxin following oral administration.

U.S. Patent No. 5,681,571 to Holmgren *et al.* proposes an immunological tolerance-inducing agent. U.S. Patent No. 5,589,384 to Lipscombe *et al.* proposes a fusion protein for use in a vaccine that employs a B subunit of an enterotoxin. PCT

Publication WO 95/08347 relates efforts to reduce or suppress the immune response of a mammal using transgenic plants.

It is an object of the present invention to provide immunomodulatory transmucosal carrier molecules, such as CTB, in food plants in order to improve the efficacy of microbial antigens expressed in food plants. To this end, it is desired to express CTB in plant tissues and to assess the efficacy of such plant material as a vaccine, either against cholera itself or as a carrier for another microbial antigen. It is another object of the invention to provide novel oral tolerization agents for prevention of such autoimmune diseases as Type I diabetes (Zhang *et al.*, 1991), collagen-induced arthritis (Trentham *et al.*, 1993), and multiple sclerosis (Khoury *et al.*, 1990; Miller *et al.*, 1992; Weiner *et al.*, 1993), as well as the prevention of allergic and allograft rejection reactions (Sayegh *et al.*, 1992; Hancock *et al.*, 1993). In this respect it is desired to express CTB as a carrier for an antigen associated with an autoimmune disease, so that oral tolerance may be achieved so as to prevent or mitigate the disease.

Disclosure of the Invention

The present invention is directed to producing an enterotoxin peptide covalently linked to a protein sorting signal. Thus, a DNA construct encodes a fusion protein comprising a non-toxic subunit of an enterotoxin and a signaling peptide. Representative of the enterotoxin subunit is the B subunit of cholera toxin (the CTB subunit). A preferred signaling peptide is a microsomal retention signal, e.g., ER signal, such as the amino acid sequence Ser-Glu-Lys-Asp-Glu-Leu, which is preferably located at the C-terminus of the fusion protein.

A DNA construct of the present invention is operably linked to one or more regulatory sequences, e.g., promoter, enhancer, polyadenylation or termination signal, to effect expression of the coding sequence for the fusion protein. Since the DNA construct is preferably expressed in a plant, the promoter must be functional in the plant. A preferred promoter is selected from cauliflower mosaic virus 35S (CaMV 35S), tomato E8, patatin (such as 1B33), ubiquitin, mannopine synthase P1, mannopine synthase P2, *A. tumefaciens* gene 5, rice actin 1, *B. mori* cytoplasmic actin, and tandem repeats thereof. An enhancer, such as cucumber mosaic virus *ires* and tobacco etch virus translation enhancer (TEV), can also be operably linked to a nucleotide sequence of the invention.

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In order to effect transfer and preferably integration of an instant DNA construct into the nuclear genome of a plant, a DNA construct of the present invention is typically provided with flanking right and left T-DNA border regions of *Agrobacterium tumefaciens*. These border regions permit transfer of the DNA construct from a suitable cloning vector to a plant cell upon activation of the *vir* genes of *A. tumefaciens*.

In another aspect of the invention, a DNA construct further comprises a nucleotide sequence encoding an antigenic polypeptide, which is positioned between an enterotoxin subunit and a signaling peptide. The antigenic polypeptide is not positioned at the C-terminus of the fusion so as not to interfere with operation of the signal peptide. Moreover, it is found that the enterotoxin subunit is preferably located at the N-terminus of the protein, perhaps to facilitate interaction with ganglioside receptors. The antigenic polypeptide region encoded by the DNA construct is typically a mammalian (such as muc-1), bacterial, viral, or fungal peptide sequence, e.g., a coat protein, which is sufficiently large and properly folded so as to effect an immunogenic response in a host animal. Notably, the enterotoxin subunit of the fusion protein can act as a carrier for the antigenic polypeptide, while further providing an adjuvant effect.

A further aspect of the invention is a transgenic plant cell, regenerated plant, seed, or progeny of a cell transformed with an instant DNA construct. Generally, the DNA construct is integrated into the nuclear genome of the cell; however, the construct can exist as a nuclear episome or extrachromosomal DNA. Preferably, the cell, seed or plant is of an edible variety, such as tobacco, potato, tomato, banana, soybean, pepper, spinach, carrot, maize, corn, wheat, rye, and rice.

A method of transforming a plant cell with a DNA construct of the present invention is also contemplated, which is preferably performed by *A. tumefaciens* transformation or by microparticle bombardment. Once transformed, the plant cell can be regenerated to an adult plant by conventional techniques, and an immunogenic amount of fusion protein can be expressed in the transgenic plant by cultivating and maintaining it under conditions effective to express the fusion protein. Optimally, expression can be effected through use of a tissue-specific promoter or one having enhanced effectiveness at a desired stage of differentiation or in the presence of a chemical agent.

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In a preferred embodiment, the CTB coding sequence is operably linked (in-frame) to a nucleotide sequence encoding an ER retention signal (Munro and Pelham, 1987). The resultant fusion is transferred into potato leaf explants by an *Agrobacterium tumefaciens* mediated stable transformation method (de Block, 1988). The transgenic potato plants are analyzed for production of CTB retaining native antigenicity, oligomeric structure and G_{M1}-ganglioside binding capacity.

A DNA construct of the invention can further comprise a selectable marker gene, such as one that codes for antibiotic resistance or a visualizable protein. A representative visualizable protein is a luciferase, green fluorescent protein, glucuronosidase or β-galactosidase. A DNA construct can also comprise a nucleotide sequence encoding a leader sequence at the N-terminus of the fusion protein.

An expression vector of the invention preferably also contains an *E. coli* origin of replication to permit the use of conventional cloning techniques. The expression vector can also comprise an *A. tumefaciens* origin of replication to permit replication in this host. Accordingly, strains of *E. coli* and of *A. tumefaciens* transfected with an expression vector of the invention are contemplated. An *A. tumefaciens* strain may further comprise a helper Ti plasmid.

A central aspect of the present invention is a transgenic plant cell transformed with the DNA construct of the invention, particularly one in which the DNA construct is integrated into the nuclear genome of the cell. The plant cell can be from one of the aforementioned plants. Similarly, a transgenic plant seed transformed with the DNA construct is contemplated.

A transgenic plant, such as one regenerated from a transformed cell or progeny from a seed, is contemplated. The transgenic plant is transformed with a DNA construct of the invention. Usually, a DNA construct is integrated into the nuclear genomes of each cell of the plant. Preferably, the transgenic plant is of an edible variety, such as tobacco, potato, tomato, banana, soybean, pepper, spinach, carrot, maize, corn, wheat, rye, and rice.

A method of transforming a plant cell with a DNA construct of the invention comprises contacting the plant cell with a strain of *A. tumefaciens* under conditions effective to transfer and integrate the construct into the nuclear genome of the cell. A

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transgenic plant can thereby be regenerated from the plant cell. An alternative method, which is preferred for monocots, comprises subjecting the plant cell to microparticle bombardment with solid particles loaded with a DNA construct under conditions effective to transfer and integrate the construct into the nuclear genome of the cell. A method of producing an immunogen in a plant is thereby afforded. The method comprises cultivating a transgenic plant of the invention under conditions effective to express an instant fusion protein.

As used herein, the term "operably linked" refers to the respective coding sequence being fused in-frame to a promoter, enhancer, termination sequence, and the like, so that the coding sequence is faithfully transcribed, spliced, and translated, and the other structural features are able to perform their respective functions.

A heterologous nucleotide sequence of the present invention can be provided as its wild-type sequence, or as a synthetic sequence, such as a "plant-optimized" sequence. A nucleotide sequence having a high degree of homology to these sequences, so that the encoded amino acid sequence remains substantially unchanged, is contemplated. In particular, sequences at least 80%, more preferably 90%, homologous with an aforementioned nucleotide sequence are contemplated. Notably, only those epitopes of an expressed antigenic protein essential for generating the desired immune response need be present in the molecule. Accordingly, C- and/or N-terminal fragments, including portions of fusion proteins, presenting the essential epitopes are contemplated within the invention.

Preferred heterologous proteins for use with the present invention include reporter molecules, such as firefly luciferase, glucuronosidase (GUS), green fluorescent protein (GFP) compounds, and enhanced versions thereof, particularly for use in optimizing the parameters of this expression system. Preferred antigenic proteins and polypeptides include shigatoxin B (StxB), staphylococcus enterotoxin B (SEB), lethal toxin B (LT-B), Norwalk virus capsid protein (NVCP), and hepatitis B surface antigen (HBsAg).

A nucleotide sequence of the invention is preferably operably linked at its 3' end to a plant-functional termination sequence. Preferred termination sequences include nopaline synthase (*nos*), vegetative storage protein (*vsp*), and protease inhibitor 2 (*pin2*) termination sequences.

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As used herein, the term "vector", and the like, refers to a nucleic acid construct capable of self-replication. Such a vector includes a plasmid, bacteria transformed with plasmids, phage vectors, cosmids, and bacterial and yeast artificial chromosomes. Generally, a vector of the present invention will be a plasmid, whether it is present *in vitro*, in *E. coli*, in *A. tumefaciens*, or as a nuclear episome of a plant. Suitable techniques for assembling the instant structural components into an expression cassette or replicon are described by Maniatis *et al.* (1982).

A strain of bacteria, such as *E. coli*, can be transfected with an expression vector of the present invention in order to grow/amplify an instant expression cassette. The *E. coli* can also be mated with *A. tumefaciens* to introduce the vector therein, where it can reside intact as a shuttle vector. A helper Ti plasmid in the *A. tumefaciens* can provide the *vir* genes necessary to transfer the T-DNA directly from the shuttle vector to the plant cell. Alternatively, the vector can undergo homologous recombination with a tumor-inducing (Ti) plasmid and exchange the instant cassette for the T-DNA of the Ti plasmid.

Transformation methods

Methods of gene transfer into plants include use of the *A. tumefaciens* --Ti plasmid system. The tumor-inducing (Ti) plasmids of *A. tumefaciens* contain a segment of plasmid DNA called transforming DNA (T-DNA), which integrates into the plant host genome. First, a plasmid vector is constructed that replicates in *E. coli*. This plasmid contains the DNA encoding the protein of interest (an antigenic protein in this invention) and this DNA is flanked by T-DNA border sequences, which define the points at which the DNA integrates into the plant genome. Usually a gene encoding a selectable marker (such as a gene encoding resistance to an antibiotic such as kanamycin) is also inserted between the left border (LB) and right border (RB) sequences. The expression of this gene in transformed plant cells gives a positive selection method to identify those plants or plant cells having an integrated T-DNA region. Second, the plasmid is transferred to *Agrobacterium*. This can be accomplished via a conjugation mating system, or by direct uptake of plasmid DNA by the *Agrobacterium*. For subsequent transfer of the T-DNA to plants, the *Agrobacterium* strain utilized must contain a set of inducible virulence (*vir*) genes, which are essential for T-DNA transfer to plant cells.

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The *A. tumefaciens* gene transfer system mentioned above is the etiologic agent of crown gall, a disease of a wide range of dicotyledons and gymnosperms [DeCleene, M. et. al., Bot. Rev. 42, 389 (1976)], that results in the formation of tumors or galls in plant tissue at the site the infection. The *Agrobacterium* system has been developed to permit routine transformation of a variety of plant tissue [see, e.g., Schell, J. *et al.*, *Bio/Technology* 1, 175 (1983); Chilton, M-D, *Scientific American* 248, 50 (1983)]. Representative tissues transformed in this manner include tobacco [Barton, K. *et al.*, Cell 32, 1033 (1983)]; tomato [Fillatti, J. *et al.*, *Bio/Technology* 5, 726 (1987)]; sunflower [Everett, N. *et al.*,. *Bio/Technology* 5, 1201 (1987)]; cotton [Umbeck, P. *et al.*, *Bio/Technology* 5, 263 (1987)]; rapeseed [Pua, E. *et al.*, *Bio/Technology* 5, 815 (1987)]; potato [Facciotti D. *et al.*, *Bio/Technology* 3, 241 (1985); poplar [Pythoud, F. *et al.*, *Bio/Technology* 5, 1323 (1987); and soybean [Hinchee, M. *et al.*, *Bio/Technology* 6, 915 (1988)]. Other plants can be transformed by routine extensions or modifications of these methods.

Multiple choices of *Agrobacterium* strains and plasmid construction strategies can be used to optimize genetic transformation of plants. For instance, *A. tumefaciens* may not be the only *Agrobacterium* strain used. Other *Agrobacterium* strains such as *A. rhizogenes* may be more suitable in some applications. *A. rhizogenes*, which incites root hair formation in many dicotyledonous plant species, carries a large extra-chromosomal element called an Ri (root-including) plasmid, which functions in a manner analogous to the Ti plasmid of *A. tumefaciens*. Transformation using *A. rhizogenes* has developed analogously to that of *A. tumefaciens* and has been successfully utilized to transform, for example, alfalfa, [Sukhapinda, K. *et al.*, *Plant Mol. Biol.* 8, 209 (1987)].

Methods of inoculation of the plant tissue vary depending upon the plant species and the *Agrobacterium* delivery system. A convenient approach is the leaf disc procedure which can be performed with any tissue explant that provides a good source for initiation of whole plant differentiation. The addition of nurse tissue may be desirable under certain conditions. Other procedures such as *in vitro* transformation of regenerating protoplasts with *A. tumefaciens* may be followed to obtain transformed plant cells as well.

Several so-called "direct" gene transfer procedures have been developed to transform plants and plant tissues without the use of an *Agrobacterium* intermediate.

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Plant regeneration from protoplasts is a particularly useful technique [Evans, D. A. *et al.*, Handbook of Plant Cell Culture 1, 124 (1983)]. When a plant species can be regenerated from protoplasts, direct gene transfer procedures can be utilized and transformation is not dependent on the use of *A. tumefaciens*. In the direct transformation of protoplasts the uptake of exogenous genetic material into a protoplast may be enhanced by use of a chemical agent or electric field. The exogenous material may then be integrated into the nuclear genome.

Early work has been conducted in the dicot *Nicotiana tabacum* (tobacco) where it was shown that the foreign DNA was incorporated and transmitted to progeny plants [Paszkowski, J. *et al.*, *EMBO J*, 3: 2717 (1984); Potrykus, I. *et al.*, *Mol. Gen. Genet.* 199: 169 (1985)]. Monocot protoplasts have also been transformed by this procedure: for example, *Triticum monococum* [Lorz H. *et al.*, *Mol. Gen. Genet.* 199: 178 (1985)]; *Lolium multiflorum* (Italian ryegrass), Potrykus, I. et. al., *Mol. Gen. Genet* 199, 183 (1985); maize [Rhodes, C., *et al.*, *Bio/Technology* 5, 56 (1988)]; and Black Mexican sweet corn [Fromm, M. *et al.*, *Nature* 319, 719 (1986)]. Other plants that have been regenerated from protoplasts include rice [Abdulah, R. *et al.*, *Bio/Technology* 4, 1987 (1987)]; rapeseed [Kansha, *et al.*, *Plant Cell Reports* 5, 101 (1986)]; potato [Tavazza, R. *et al.*, *Plant Cell Reports* 5, 243 (1986)]; eggplant, Sihachaki, D. *et al.*, *Plant Cell, Tissue, Organ Culture* 11, 179 (1987); and cucumber [Jia, S-R., *et al.*, J. Plant Physiol. 124, 393 (1986)]. Methods for directly transforming protoplasts of other varieties are evident.

Introduction of DNA into protoplasts of a plant can be effected by treatment of the protoplasts with an electric pulse in the presence of the appropriate DNA in a process called electroporation. In this method, the protoplasts are isolated and suspended in a mannitol solution. Supercoiled or circular plasmid DNA is added. The solution is mixed and subjected to a pulse of about 400 V/cm at room temperature for less than 10 to 100 microseconds. A reversible physical breakdown of the membrane occurs to permit DNA uptake into the protoplasts.

DNA viruses have been used as gene vectors in plants. A cauliflower mosaic virus carrying a modified bacterial methotrexate-resistance gene was used to infect a plant. The foreign gene was systematically spread in the plant [Brisson, N. *et al.*, *Nature*

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310, 511 (1984)]. The advantages of this system are the ease of infection, systematic spread within the plant, and multiple copies of the gene per cell.

Liposome fusion has also been shown to be a method for transformation of plant cells. In this method, protoplasts are brought together with liposomes carrying the desired gene. As membranes merge, the foreign gene is transferred to the protoplasts [Dehayes, A. *et al.*, *EMBO J.* 4, 2731 (1985)].

Polyethylene glycol (PEG) mediated transformation has been carried out in *N. tabacum* (a dicot) and *Lolium multiflorum* (a monocot). It is a chemical procedure of direct gene transfer based on synergistic interaction between Mg^{2+}, PEG, and possibly Ca^{2+} [Negrutiu, R. *et al.*, *Plant Mol. Biol.* 8, 363 (1987)]. Alternatively, exogenous DNA can be introduced into cells or protoplasts by microinjection. A solution of plasmid DNA is injected directly into the cell with a finely pulled glass needle.

A recently developed procedure for direct gene transfer involves bombardment of cells by microprojectiles carrying DNA [Klein, T. M. *et al.*, *Nature* 327, 70 (1987)]. In this "biolistic" procedure, tungsten or gold particles coated with the exogenous DNA are accelerated toward the target cells. At least transient expression has been achieved in onion. This procedure has been utilized to introduce DNA into Black Mexican sweet corn cells in suspension culture and maize immature embryos and also into soybean protoplasts [Klein, T. M. *et al.*, *Bio/Technology* 6, 559 (1988)]. Stably transformed cultures of maize and tobacco have been obtained by microprojectile bombardment. Stably transformed soybean plants have been obtained by this procedure [McCabe, D. E. *et al.*, *Bio/Technology* 6, 923 (1988)].

Immunization methods

A method of inducing immunity to an enterotoxin in a mammal or avian is thereby provided. Such method entails the animal consuming an immunizing effective amount of an edible part of the transgenic plant. Hence, immunity to cholera is effected when the enterotoxin subunit is the CTB subunit. Antibiotic resistant avians can be particularly advantaged.

Similarly, a method of effecting or boosting immunity of a mammal or avian to a pathogen can be effected, particularly whenever the fusion protein contains an antigenic (heterologous) peptide sequence capable of eliciting antibodies to the pathogen. Such a

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method entails the mammal or avian consuming an adjuvant effective amount of an edible part of a transgenic plant, and subjecting the animal to a live, dead, or attenuated amount of said pathogen, or antigenic portion thereof. In this aspect, the antigenic portion is provided by the antigenic polypeptide of the fusion protein. Preferably, the antigenic polypeptide can assemble as an antigenic complex or particle, and the subunit acts as a carrier for the antigenic polypeptide.

In a preferred embodiment, the use of transgenic food plants for the production and delivery of oral vaccine against microbial enteropathogens and their toxins is investigated. A cholera toxin (the prototypical diarrhea-inducing enterotoxin) is used as a model system. The oral immunization of CD-1 mice by transgenic potato plants transformed with the CTB coding sequence is studied and the levels of serum and mucosal anti-cholera toxin antibodies are determined. The efficacy of an edible vaccine against *V. cholerae* enterotoxin in mammalian cell culture and in mouse intestine is evaluated.

Brief Description of the Figures

Fig. 1 depicts the structure of the plant expression vector pPCV701FM4-CTB:SEKDEL. The following four genes are located within the T-DNA sequence flanked by the right and left border (RB and LB) 25 bp direct repeats required for integration of the T-DNA into plant genomic DNA: (1) 393 bp CTB:SEKDEL coding sequence under control of the *mas* P2 promoter; (2) the bacterial luciferase AB fusion gene (*luxF*) under control of the *mas* P1 promoter as a detectable marker; (3) an NPT-II expression cassette for resistance to kanamycin in plants; (4) a β-lactamase cassette for resistance to ampicillin in *E. coli* and carbenicillin in *A. tumefaciens*. The g7pA polyadenylation signal is from the *A. tumefaciens* T_L-DNA gene 7; the OcspA polyadenylation signal is from the octopine synthase gene; Pnos is the promoter of the nopaline synthase gene; g4pA is the polyadenylation signal from T_L-DNA gene 4; OriT is the origin of transfer derived from pRK2; OriV is the wide host range origin of replication for multiplication of the plasmid in *A. tumefaciens* derived from pRK2; and Ori pBR322 is the replication origin of pBR322 for maintenance of the plasmid in *E. coli*.

Fig. 2 shows the luciferase activity in transformed potato leaf tissues. Bacterial luciferase activity was detected in leaves of six kanamycin-resistant potato plants (Nos

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1-6) by low-light image analysis after induction on high auxin medium for 48 h (columns A and B in duplicate). Arrows indicate leaves with relatively high light intensities. No luciferase activity was detected in leaves of six untransformed potato plants (Nos 7-12, columns C and D in duplicate). Photon detection period was 5 min.

Fig 3 shows the detection of the CTB fusion gene in genomic DNA of transformed potato leaves. Lane 1 is a 1 kb DNA ladder (Gibco Life Technologies). DNA templates used for PCR amplification reaction of the CTB gene were as follows: lane 2, pPCV701FM4-CTB:SEKDEL plasmid DNA (10 ng); lane 3, untransformed potato plant genomic DNA (500 ng); lane 4-6, transformed potato plant genomic DNA (500 ng) from plants No. 1, No. 3, and No. 4.

Fig. 4 shows immunoblot detection of plant CTB protein. Auxin-induced leaf callus tissues derived from transgenic potato plant No. 4 were analyzed for the expression of multimeric CTB protein, which dissociated to monomers by heat treatment. Fig. 4A: Multimeric CTB. Lane 1, 100 ng bacterial CTB; lane 2, 100 ng bacterial CTB mixed with total protein (100 μg) from untransformed potato plant leaf callus tissue; lane 3, total protein (100 μg) from untransformed potato plant leaf callus tissue; lane 4, total protein (100 μg) from No. 4 transgenic potato plant leaf callus tissue. Fig. 4B: Monomeric CTB. Lane 1, 100 ng bacterial CTB multimer (M_r ~ 45 kDa) partially dissociated to monomer (M_r ~ 12 kDa); lanes 2 (boiled) and 3 (unboiled), total protein (100 μg) from untransformed potato plant callus tissue; lanes 4 (boiled) and 5 (unboiled), total protein (100 μg) from No. 4 transgenic potato plant leaf callus tissue. Arrow indicates the band (M_r ~ 15 kDa) corresponding to leaf callus CTB monomer. Fig. 4C: CTB in tuber tissues. Lane 1, 100 ng bacterial CTB (boiled); lane 2, total protein (150 μg) from No. 4 transgenic potato plant microtuber tissue (boiled); lane 3, total protein (150 μg) from untransformed potato plant microtuber tissue (boiled); lanes 4-6, identical to lanes 1-3, respectively, except samples were not boiled prior to SDS-PAGE. Arrow indicates microtuber CTB monomer (M_r ~15 kDa).

Fig. 5 illustrates the determination of CTB protein levels in transgenic potato plants using ELISA. The ELISA detection of plant-synthesized CTB using a variety of transgenic plant protein concentrations indicates that CTB protein levels in induced leaf tissues from transgenic potato plant No. 4 reaches a maximum 0.3% of total soluble plant

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protein. CTB protein levels (% CTB) were plotted against dilutions of plant homogenate. Error bars indicate SE.

Fig. 6 compares the G_{M1} binding ability of plant-derived CTB, bacterial CTB, and bacterial CTX. The ELISA assay was performed by coating microtiter plates with either G_{M1}-monosialoganglioside, sucrose, galactose, or BSA as CTB or CTX receptor molecules (300 ng/well). For binding the plant-derived CTB (A), a transformed plant tissue homogenate containing approximately 30 ng of recombinant CTB in 10 μg of total soluble protein was used per well. For binding bacterial CTB (B) and CTX (C), 30 ng of each molecule was mixed with untransformed plant homogenate containing 10 μg of total soluble protein per well. Relative binding affinity of plant CTB, bacterial CTB, and CTX for receptor molecules was expressed as relative light units (RLU).

Fig. 7A illustrates the CTB plant expression vector pPCV701FM4-CTB:SEKDEL. The T-DNA sequence flanked by right and left borders (RB and LB) contains the *luxF*/CTB:SEKDEL expression cassette containing the bi-directional mannopine synthase (*mas*) P1 and P2 promoters (Koncz, C. *et al.*, 1987). The *luxF* gene is a detectable marker for agrobacteria and plants (Escher, A., *et al.*, 1989; Langridge, W., *et al.*, 1989). The NPT II expression cassette containing the nopaline synthase promoter provides kanamycin resistance in plants, and the β-lactamase (Bla) expression cassette provides ampicillin resistance in *Escherichia coli* and carbenicillin resistance in *Agrobacterium tumefaciens*. The CTB fusion gene contains the DNA sequence encoding the CTB leader peptide at the 5' end and an ER retention signal (SEKDEL) at the 3' end. The g7pA, g4pA and OcspA sequences are polyadenylation signals from *A. tumefaciens* T_L-DNA gene 7, gene 4, and the octopine synthase gene, respectively. Ori pBR is the origin of replication from plasmid pBR322. Fig. 7B shows the immunoblot detection of CTB protein in a transgenic potato plant. Microtuber homogenate from a transformed potato plant (lane 3; 100 μg total soluble protein/lane) revealed the chimeric CTB pentamer (~ 50 kDa), which was slightly larger than the bacterial CTB pentamer (~ 45 kDa), (lane 1; 100 ng). Untransformed potato tuber proteins did not react with anti-CT antibody (lane 2; 100 μg total soluble protein/lane). Fig. 7C shows the effects of boiling tuber tissues on CTB pentamer dissociation. Potato tuber tissue slices (~ 300 mg) were boiled for increasing time intervals in a plant extraction buffer (v:w = 1:1.5) and the

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amount of CTB (G_{M1}-ganglioside binding form) was determined by G_{M1}-ELISA. The amount of CTB in the tuber homogenate prior to boiling (time 0) was considered to be 100%. The increase in CTB amount detected after 0.5 min boiling may indicate enhanced extraction of plant soluble protein due to tissue softening. Transgenic potato tissues became soft after 3 min boiling which corresponded to CTB levels of 50%. Error bars indicate standard errors of the mean for four separate measurements.

Fig. 8A illustrates anti-CTB antibody endpoint titer determination. An example of an endpoint (E) titer measurement using a fecal IgA on day 38. The same general method was used for both fecal and serum E titer determination for all isotypes of antibody in serum and fecal samples. Baseline RLU: background signal from the enzyme-substrate reaction alone. R^2: regression coefficient. The thin line is an extrapolation of the sample value to the baseline for determination of the E titer. Fig. 8B shows serum anti-CTB antibody titers. The E titers of three isotypes (B1, IgG; B2, IgA; B3, IgM) of serum anti-CTB antibody are expressed for days 35 (■)and 70 (❑). Bacterial CTB: is the serum sample obtained from mice orally immunized with 30 µg of bacterial CTB. Potato (1g) and (3g): are serum samples obtained from mice orally immunized with 1 g and 3 g of transgenic potato tissues, respectively. Negative control: serum sample derived from mice orally immunized with 1 g of untransformed potato tissues. Error bars were determined based on the fluctuations of RLU baseline values which affect the E titer. Fig. 8C shows mucosal anti-CTB antibody titers. The E titers of mucosal (fecal) anti-CTB isotypes (C1, IgA; C2, IgG) were expressed for each day of fecal sample collection. Legends in the box and error bar determination are the same as in serum antibody titers except the samples are fecal pellets.

Fig. 9 depicts an ileal loop ligation assay and the effect of oral immunization with bacterial or plant CTB on the small intestine. Fig. 9A shows ileal loops excised from un-immunized mice. Fig. 9B shows the same from an orally immunized mouse. The middleand right loops were challenged with CT (125 ng/loop). The left loop was injected with physiological saline. Fig. 9C shows the intestinal fluid accumulation per unit length of ligated ileal loops from un-immunized and immunized mice. The reduction in fluid secretion into the ileal loop in the immunized mice in comparison with un-immunized mice (*) is expressed as % protection above each bar. A significant difference in

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reduction of fluid accumulation in mice orally immunized with plant or bacterial CTB was observed in comparison with mice fed untransformed potato tissues ($p<0.05$).

Fig. 10 depicts a G_{M1}-ELISA assay of CT binding to G_{M1}-ganglioside. The G_{M1}-ELISA method described in the section on materials and methods was used to elucidate the protective mechanism of anti-CTB antibody. Approximately 35% less RLU signal was detected from G_{M1}-ganglioside-coated microtiter wells [GM1(+)] when CT was incubated with immune serum prior to assay than when CT was incubated with non-immune serum. In the absence of G_{M1}-ganglioside [GM1(−)], CT incubated with either immune or non-immune serum resulted in similar RLU levels. The RLU levels in G_{M1}-ganglioside coated and in uncoated wells cannot be compared directly as affinities of CT to G_{M1}-ganglioside and the microtiter plate plastic surface differ. Data represent mean values of RLU ± standard deviation of four individual samples.

The invention will now be described with reference to certain examples, which illustrate but do not limit it.

Examples

Example 1. Construction of plant expression vector pPCV701FM4-CTB:SEKDEL.

The plant expression vector pPCV701FM4 was derived from plasmid pPCV701 by addition of multiple cloning sites immediately downstream from the mannopine synthase (*mas*) P2 promoter. The vector was digested with *Xba*I and *Sac*I restriction endonucleases within the multiple cloning site to insert a gene encoding the cholera toxin B subunit from plasmid pRT42 containing the *ctxAB* operon (provided by Dr. J. Mekalanos, Harvard Medical School). The 5' primer: (5'-GCTCTAGAGCCACCATGATTAAATTAAAATTTGGTG-3') and the 3' primer: (5'-CTGGAGCTCATAGCTCATCTTTCTCAGAATTTGCCATACTAATTGCGG-3') were synthesized (model 394 DNA/RNA Synthesizer Applied Biosystems, Inc.) in the DNA Core Facility at Loma Linda University, with *Xba*I and *Sac*I restriction endonuclease recognition sites for amplification and cloning of the CTB:SEKDEL fusion gene construct (393 bp) in vector pPCV701FM4. The 3' primer was designed to contain a nucleotide sequence encoding a hexapeptide ER retention signal (SEKDEL) in frame with the CTB open reading frame. After PCR amplification (Perkin Elmer Gene Amp PCR System 9600) and ligation of the CTB:SEKDEL coding sequence into the plant

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expression vector to create pPCV701FM4-CTB:SEKDEL, the reaction mixture was used to transform *Escherichia coli* strain HB101 by electroporation (Gene Pulser, Bio-Rad, Inc. Hercules, CA) at a setting of 250 μFD, 200 Ω, and 2,500 V. Ampicillin resistant colonies were isolated after overnight culture at 37 °C. To confirm the presence of the correct CTB fusion gene sequence in transformed *E. coli* cells, the plasmid was isolated from individual colonies of transformants and subjected to DNA sequence analysis with the forward primer (5'-ACCAATACATTACACTAGCATCTG-3') specific for the *mas* P2 promoter and the reverse primer (5'-GACTGAGTGCGATATTATGTGTAATAC-3') specific for the gene 7 poly(A) signal in a model 373A DNA Sequencer (Applied Biosystems, Inc.).

Following confirmation of the correct CTB fusion DNA sequence, the shuttle vector was transferred into *A. tumefaciens* recipient strain GV3101 pMP90RK by the same electroporation conditions described for *E. coli* transformation. For selection of transformants, the bacteria were grown at 29 °C on YEB solid medium (beef extract 5.0 g/L, Bacto yeast extract 1.0 g/L, Bacto peptone 1.0 g/L, sucrose 5.0 g/L, $MgSO_4 \bullet 7H_2O$ 0.1 g/L) containing the antibiotics carbenicillin (100 μg /mL), rifampicin (100 μg /mL), kanamycin (25 μg/mL), and gentamycin (25 μg /mL). The plasmid was isolated from an *A. tumefaciens* transformant and transferred back into *E. coli* HB101, by electroporation to confirm by restriction endonuclease analysis, that no significant deletion had occurred in the vector. Structural confirmation of the plasmid was required because recombination events within the rec^+ *A. tumefaciens* strain could alter the T-DNA sequence. Transfer of the plasmid from *A. tumefaciens* back to the *E. coli* host was necessary because significant amounts of plasmid are difficult to isolate directly from *A. tumefaciens*. Agrobacteria carrying the plant expression vector were grown on YEB solid medium containing all antibiotics for 48 h at 29 °C and directly used for transformation of sterile potato leaf explants.

Example 2. Plant transformation

Sterile potato plants *Solanum tuberosum* cv. Bintje were grown in Magenta boxes (Sigma) or Mason jars on solid Murashige and Skoog (MS) complete organic medium

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(JRH Biosciences No. 56-750-015) containing 3.0% sucrose and 0.2% gelrite (a clear *Pseudomonas* polysaccharide solid support medium). Leaf explants were excised from the young plants and laterally bisected in a 9 cm diameter culture dish containing an overnight culture of *A. tumefaciens* suspension (2-5 x10^9 cells/mL) harboring pPCV701FM4-CTB:SEKDEL. Acetosyringone (370 μM) was added to the bacterial suspension to facilitate transformation. The explants were incubated in the bacterial suspension for 5 min, blotted on sterile filter paper, and transferred to MS solid medium, pH 5.7, containing the plant hormones auxin (0.1 μg/mL naphthalene acetic acid (NAA)) and cytokinin (1.0 μg/mL trans-zeatin). The leaf explants were incubated for 48-72 h at room temperature on MS solid medium to permit T-DNA transfer into the plant genome. The leaf explants were transferred to MS solid medium containing the antibiotics kanamycin (100 μg/mL) and claforan (400 μg/mL), for selection of transformed plant cells and for counterselection against continued *Agrobacterium* growth, respectively.

Transformed plant cells formed calli on the selective medium after continuous incubation for 2-3 weeks at 25 °C in a light room under cool white fluorescent tubes on a 12-h photoperiod regime. When transformed calli grew to 5-10 mm in diameter, the leaf tissue was transferred to MS medium containing 1.0 μg/mL trans-zeatin, 50 μg/mL kanamycin and 400 μg/mL claforan for shoot induction. After 4-5 weeks incubation, regenerated shoots were excised at the base from the calli and transferred to MS solid medium without plant hormones or antibiotics to stimulate root formation. Plantlets were obtained after about 6 weeks further growth under sterile conditions in Mason jars. The plantlets were grown into mature plants (4-6 weeks) in potting soil in the greenhouse under a 12-h photoperiod. Activity of the luciferase reporter gene was detected in leaf tissues of the putative transformed plants by low-light image analysis with a Hamamatsu Argus-100 intensified camera system (Hamamatsu Photonics, K.K., Japan).

Example 3. Detection of luciferase activity in transformed A. tumefaciens and transgenic plants.

To detect the presence of the plant expression vector in agrobacteria, bacterial luciferase gene expression under control of the *mas* P1 promoter was monitored by low-

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light image analysis (Langridge *et al.*, 1991). To perform the bioluminescent assay for bacterial luciferase, the volatile substrate N-decyl aldehyde (Sigma D-7384) was applied to a 9 cm diameter glass culture plate lid by swabbing the plate with substrate-saturated cotton. A culture plate containing bacterial colonies grown for 24-48 h on YEB solid medium, was covered with the substrate coated glass lid, and the culture plate was transferred into the photon counting chamber of the Argus-100 intensified camera system for photon counting for a period of 1-5 min.

Bacterial luciferase bioluminescence was also used to detect insertion of the T-DNA into the plant genome and to estimate the level of *mas* P2 promoter driven expression of the CTB gene by the level of *mas* P1 driven expression of the *luxF* gene. Leaves excised from putative transformants were wounded by cutting perpendicular to the central vein with a sterile scalpel blade followed by incubation of the wounded leaf tissue on MS solid medium containing NAA (5.0 mg/L) and 2,4-dichlorophenoxy acetic acid (2,4-D) (6.0 mg/L) for 48 h. Wounding and subsequent incubation on high auxin medium for several days is necessary to detect the maximum amount of gene expression from the *mas* promoters in potato plant tissues. Light emission from the wounded leaf tissues was detected as described for agrobacteria. Approximately 5-30 min exposure was required to obtain an coherent photon emission image.

Example 4. Detection of the CTB gene in transformed plant genomic DNA.

Genomic DNA was isolated from transformed potato leaf tissues as described by Doyle and Doyle (1992) with the following modification: biological grinding spheres (Boehringer Mannheim) were used instead of a mortar and pestle for grinding the plant tissues. Presence of the CTB gene was determined by PCR analysis using the oligonucleotide forward and reverse primers specific for the pPCV701FM4 vector. Transformed plant genomic DNA (500 ng) was used as a template to detect the CTB gene under the following PCR conditions: 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 60 s for a total of 30 cycles. PCR samples were separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

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Example 5. Immunoblot detection of CTB protein in transformed potato tissues.

Transgenic potato tissues were evaluated for the presence of CTB protein by immunoblot analysis using a Bio-Rad Immun-Lite Assay Kit (Bio-Rad 170-6471). Callus tissues were derived from leaf or tuber tissues incubated for 5-7 weeks on MS solid medium containing NAA (5.0 mg/L) and 2,4-D (6.0 mg/L). Tissues (~ 1 g fresh weight) were homogenized by grinding in a mortar and pestle on ice in 1.0 mL of extraction buffer (200 mM Tris-Cl, pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.05% Tween-20). The tissue homogenate was centrifuged twice at 17,000 x g in a Beckman GS-15R centrifuge for 15 min at 4 °C to remove insoluble cell debris. An aliquot of 10-20 µL of supernatant, containing 50-100 µg of total soluble protein, as determined by Bradford protein assay (Bio-Rad, Inc.), was separated by 15% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 125 V for 30-45 min in Tris-glycine buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS). Samples of the plant homogenate and purified CTB (Sigma C-9903) were either loaded directly on the gel or boiled for 3 min prior to electrophoresis.

The separated protein bands were transferred from the gel to Immun-Lite membranes by electroblotting on a semi-dry blotter (Labconco) for 60-90 min at 15 V and 100 mA. Nonspecific antibody reactions were blocked by incubation of the membrane in 25 mL of 5% non-fat dry milk in TBS buffer (20 mM Tris, pH 7.5 and 500 mM NaCl) for 1 h on a rotary shaker (40 rpm), followed by washing in TBS buffer for 5 min with gentle agitation. The membrane was incubated overnight at room temperature with gentle agitation in 30 mL of a 1:5,000 dilution of rabbit anti-cholera antiserum (Sigma C-3062) in antibody dilution buffer (TBST (TBS with 0.05% Tween-20) containing 1% non-fat dry milk) followed by washing three times in TBST buffer. The membrane was incubated for 1 h at room temperature with gentle agitation in 30 mL of a 1:10,000 dilution of mouse anti-rabbit IgG conjugated with alkaline phosphatase (Sigma A-2556) in antibody dilution buffer. The membrane was washed three times in TBST buffer as before and once with TBS buffer, followed by incubation in 20 mL of 1x substrate buffer containing 36 µL of chemiluminescent substrate CSPDJ for 5 min at room temperature

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with gentle agitation. The membrane was placed inside a household seal-a-meal bag after removing excess substrate buffer and placed in a photocassette on Kodak X-OMAT film (No. 1651454). (The membrane was also used to image chemiluminescent light intensity in both the numerical and graphic form by the Argus-100 video image analysis.) The film was subjected to 1-15 min exposure at room temperature in the dark for optimal image development. The exposed film was developed in a Kodak M35A X-OMAT Processor.

Example 6. Quantitation of CTB protein level in transgenic potato tissues.

CTB protein levels in transgenic potato plants were determined by quantitative chemiluminescent ELISA assays. A 96-well microtiter plate (Microlite™ 2, Dynatech Laboratories), loaded with 100 μL/well of selected concentrations of total soluble potato protein in bicarbonate buffer, pH 9.6 (15 mM Na_2CO_3, 35mM $NaHCO_3$) was incubated overnight at 4 °C. The plate was washed three times in PBST (phosphate buffered saline (PBS) containing 0.05% Tween-20). The background was blocked by incubation in 1% bovine serum albumin (BSA) in PBS (300 μL/well) at 37 °C for 2 h followed by washing three times with PBST. The plate was incubated in a 1:8,000 dilution of rabbit anti-cholera toxin antibody (Sigma C-3062), (100 μL/well) for 2 h at 37 °C, followed by washing the wells three times with PBST. The plate was incubated with a 1:80,000 dilution of anti-rabbit IgG conjugated with alkaline phosphatase (Sigma A-2556), (100 μg/well) for 2 h at 37 °C and washed three times with PBST. The plate was finally incubated with 100 μL/well of Lumi-Phos® Plus (Lumigen, Inc. P-701) for 20 min at 37 °C to maximize the reaction rate. The plate was cooled to room temperature before the enzyme-substrate reaction was measured in a Microlite™ ML3000 Microtiter® Plate Luminometer (Dynatech Laboratories), operated according to the manufacturer's instructions.

Alternatively, chemiluminescent light intensities of the enzyme-substrate reaction from bacterial and plant CTB protein bands blotted on the Immun-Lite membranes after SDS-PAGE were quantified by the Argus-100 Data Analysis Program (Hamamatsu Photonics, K.K.). The numeric value of the total light intensity of the area defined by a

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rectangular window surrounding the plant CTB protein band was compared with the bacterial CTB band, and the amount of plant CTB was estimated based on the known amount of bacterial CTB.

Example 7. CTB-G_{M1} binding assay.

A G_{M1}-ELISA assay was performed to determine the affinity of plant derived CTB for G_{M1}-ganglioside. The microtiter plate was coated with monosialoganglioside-G_{M1} (Sigma G-7641) by incubating the plate with 100 µL/well of G_{M1} (3.0 µg/mL) in bicarbonate buffer, pH 9.6 (15 mM Na_2CO_3, 35mM $NaHCO_3$) at 4 °C overnight. Alternatively, the wells were coated with 100 µL/well of BSA, sucrose, or galactose (3.0 µg/mL each) as controls. The plates were incubated with transformed plant total soluble protein, bacterial CTB (Sigma C-9903), or CTX (Sigma C-8052) in PBS (100 µL/well) overnight at 4 °C. The remainder of the procedure was identical to the ELISA described above.

Example 8. Construction of transgenic potato plants producing CTB:SEKDEL fusion peptide.

The CTB coding sequence including its putative leader peptide (which is absent in mature cholera toxin B subunit in *V. cholerae*), was PCR amplified from the *ctxAB* operon in the plasmid pRT42 (provided by Dr. J. Mekalanos). The 3' PCR primer contained an oligonucleotide sequence encoding a hexapeptide endoplasmic reticulum (ER) retention signal (SEKDEL) in frame with the CTB gene. The amplified DNA fragment containing the CTB:SEKDEL fusion gene was cloned into plant transformation vector pPCV701FM4 (Koncz, C., *et al.*, 1987). The resultant plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 pMP90RK by electroporation (Koncz, C., *et al.*, 1987). Potato plants (*Solanum tuberosum* cv. Bintje) were transformed with agrobacteria harboring pPCV701FM4-CTB:SEKDEL as previously described above.

Example 9. Immunization Antigens.

Purified cholera holotoxin and its B subunit were purchased from Sigma Chemical

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Co. (St. Louis, MO). Immunization was performed with CTB. Cholera holotoxin was used in mouse ileal loop ligation and *in vitro* toxin neutralization assay experiments. Potato tuber and leaf callus tissues producing approximately 0.3% of total soluble protein as CTB pentamers were used for oral immunization of CD-1 mice.

Example 10. Laboratory Mice and Cell Lines.

Adult female CD-1 mice were purchased from Charles River Co. (Charles River, MA). and were maintained in the University animal care facility. Sex and age-matched, untransformed potato placebo-immunized control animals were used. African Green Monkey kidney cells (Vero cells) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA) and grown as monolayers at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 5% fetal calf serum (FCS) in a 5% CO_2 atmosphere.

Example 11. Oral immunization with CTB.

Mice were fed *ad libitum* transgenic (microtuber or leaf callus) potato tissues containing approximately 30 µg CTB/g fresh weight, previously determined by CL-ELISA and immunoblot experiments with transformed plant tissue homogenates as described above. Mice were fed four times on days 0, 6, 17, 24 with a final booster dose on day 65. A group of 10 mice were fed with 1 g of transgenic potato tissues, and a group of 8 mice were fed with 3 g of transgenic potato tissues. Using the same feeding schedule, 1 g of untransformed potato plant tissues were fed to 5 mice as a negative control; a group of 8 mice were given 30 µg of bacterial CTB in sodium bicarbonate buffer (350 mM), pH 8.5 by oral feeding tube (gavage) as a positive control.

Example 12. Serum and fecal sample preparation.

For serum antibody titer determinations, animals were bled on days 35 and 70 of the experiment. Fecal pellets were collected on days 25, 28, 31, 38, 45, 65, and 70 of the experiment to determine the presence of mucosal antibodies (IgA and IgG) secreted in response to CTB ingestion. Fecal antibodies were detected according to the co-proantibody isolation method described by de Vos and Dick.

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Example 13. Chemiluminescent ELISA.

One hundred microliters of bacterial CTB diluted in bicarbonate buffer (pH 9.6) to 5.0 μg/mL was absorbed onto Microlite™ 2 microtiter plate wells (Dynatech Laboratories, Burlington, MA.) overnight at 4 °C. The plates were washed with PBS-0.05% Tween-20 and blocked with 1% BSA in PBS for 2 h at 37 °C. Serial dilutions of serum or fecal extract were added to the wells (100 μL/well) and incubated overnight at 4 °C. Anti-mouse antibody [1:20,000 dilution of anti-mouse IgG (Sigma A-3688), 1:20,000 IgA (Sigma A-4937), or 1:30,000 IgM (Sigma A-9688)] diluted in PBS containing 0.5% BSA was added (100 μL/well), and incubated for 2 h at 37 °C. The wells were washed and incubated with the chemiluminescent substrate, Lumi-Phos® Plus, (100 μL/well) (Lumigen Inc., Southfield, MI.) for 20 min at 37 °C. The plates were read in a Microlite™ ML3000 Microtiter® Plate Luminometer (Dynatech Laboratories).

Example 14. Endpoint titer measurement.

Serum and fecal endpoint titers were determined as described elsewhere (Jackson, R., *et al.*, 1996). Briefly, serial dilutions of serum or fecal extracts from immunized or unimmunized mice were transferred in duplicate into microtiter plates for CL-ELISA. Background RLU from the fecal or serum sample of unimmunized mice was subtracted from the RLU of samples from immunized mice. The resultant RLU was plotted on a $\log_{10}$ scale against two-fold dilutions ($\log_2$) of the samples. The graphic data were extrapolated for three groups of immunized mice to the level of 0.5 RLU which was the nonspecific background signal generated from the enzyme-substrate reaction alone in this assay system.

Example 15. Vero-cell based CT neutralization assay.

Vero cells were grown at 37 °C in DMEM supplemented with 5% FCS in a 5% CO_2 atmosphere. The concentrations of CT required for a cytotoxic response were determined initially by adding serial dilutions of CT to Vero cell monolayers (Stavric, S., *et al.*, 1978). The changes in cellular morphology (refractile, thick-walled with several filamentous tendrils) were scored as 0, 1, 2, 3, 4, or 5, corresponding roughly to 0, < 25,

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25–50, 50–75, 75–90, or > 90% cells affected. A final concentration of 25–30 ng CT/mL, was found to give scores above 3 and was used for toxin neutralization assays. The relative efficacy of toxin neutralization of antisera was assayed by incubating 100 µL of two-fold dilutions of pooled serum in PBS prepared from immunized or unimmunized mice with 25 ng CT for 1 h at 35 °C. Prior to addition of the toxin-antiserum mixture to confluent monolayers, the growth medium was replaced with 1 mL of fresh medium containing 5% FCS, followed by 1 h incubation at 37 °C in a 5% CO_2 atmosphere. The toxin-antiserum mixture was added to the cell monolayers and cytotoxic responses were observed after 20 h incubation at 37 °C.

Example 16. Mouse ileal loop ligation assay.

A mouse ileal loop ligation experiment was conducted essentially as described elsewhere (Punyashthiti and Finkelstein, 1971) on day 70 of the CTB oral immunization experiment. Briefly, animals were starved for 48 h and three equal loops 2 to 3 cm in length were ligated. Cholera toxin (125 ng in 30 µl physiological saline) was injected into each of the two loops, and the third loop was injected with saline only. After 24 h incubation the three consecutive ileal loops excised and were punctured to measure the fluid volume and the length of the empty loops. Student's *t*-test was used to determine the significance of fluid reduction between control mice and immunized mice.

Example 17. Antiserum-mediated inhibition of CT binding to G_{M1}-ganglioside by G_{M1}-ELISA.

A 96-well microtiter plate was coated with 100 µL/well of G_{M1}-ganglioside (3.0 µg/mL) (Sigma G-7641) in bicarbonate buffer, pH 9.6 and incubated at 4 °C overnight. Pooled serum (100 µL) from mice fed 1 g of transgenic potato tissues, or from mice fed 1 g of untransformed potato tissues was mixed with 2.5 ng of CT and incubated at 35 °C for 1 h. The serum-CT mixture was loaded into the G_{M1}-ganglioside-coated or uncoated wells and the plate was incubated at 4 °C overnight. Washing, blocking, and chemiluminescent substrate addition steps were described in the section for chemiluminescent ELISA. Rabbit anti-CT antibody (1:5,000 dilution) (Sigma C-3602)

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and anti-rabbit IgG antibody (1:50,000 dilution) (Sigma A-2556) were used as primary and secondary antibodies, respectively.

RESULTS AND DISCUSSION

Plant expression vector harboring CTB:SEKDEL fusion gene

The CTB:SEKDEL fusion gene was inserted into the plant expression vector pPCV701FM4 resulting in pPCV701FM4-CTB:SEKDEL (Fig. 1). Plant expression vector pPCV701FM4 harbors a 430 bp DNA fragment containing the *A. tumefaciens* bi-directional mannopine synthase (*mas* P1, P2) promoters (Koncz *et al.*, 1987). The P1 promoter is fused to the bacterial luciferase reporter gene (*luxF*) (Escher *et al.*, 1989), and the P2 promoter is linked to the CTB:SEKDEL fusion gene. In addition, the plant expression vector contains the β-lactamase gene, which confers ampicillin resistance in *E. coli* and carbenicillin resistance in *A. tumefaciens*. The neomycin phosphotransferase II gene (NPT-II) linked to the nopaline synthase (NOS) promoter provides selection for transformed plant cells. The oligonucleotide sequence surrounding the translation initiation codon of the CTB gene was changed to a preferred nucleotide context for translation in eukaryotic cells (Kozak, 1981), and a putative Shine-Dalgarno sequence (AGGA) present in the *ctxAB* operon in plasmid pPT42 was also removed. The DNA fragment encoding the 21-amino acid leader peptide of the CTB protein was retained to direct the newly synthesized CTB protein into the lumen of the ER. An oligonucleotide sequence encoding the ER retention signal (SEKDEL) and using codon usage favored in potato was inserted at the 3′ end of the coding sequence of the CTB gene to sequester CTB protein within the lumen of the ER. The CTB:SEKDEL fusion gene was inserted into the multiple cloning site immediately downstream of the *mas* P2 promoter.

Detection of luciferase activities from putative transgenic potato plants

After *Agrobacterium*-mediated transformation of potato leaf explants with the plant expression vector pPCV701FM4-CTB:SEKDEL, six independent kanamycin-resistant plants were regenerated. All of the plants were found to express luciferase activities (Fig. 2, rows 1-6 AB) above background levels from untransformed plants (Fig. 2, rows 7-12 CD). Thus, 100% of kanamycin-resistant plants were found to express luciferase activities in this plant transformation experiment. However, in additional

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potato plant transformation experiments using lower levels of kanamycin for selection, we observed transformation efficiencies as low as 5% (2 luciferase positive plants out of 40 kanamycin-resistant plants). Potato plant transformation efficiencies were found to vary considerably between experiments, suggesting the possibility that differences in aspects of the transformation method such as physiological state of leaf explants and the growth state of the agrobacteria used for transformation may result in substantial differences in transformation efficiency. In addition, the number of kanamycin-resistant plants regenerated per transformation experiment varied dramatically ranging from 0 to 200 per 50 leaf explants transformed. Therefore, the presence of a convenient detectable marker gene, such as luciferase, in addition to selectable marker gene significantly enhances the process of screening large numbers of antibiotic resistant putative transformants.

PCR detection of CTB fusion gene in transgenic potato plants

Three transformed potato plants showing high luciferase activities were analyzed for the presence of the CTB:SEKDEL fusion gene in genomic DNA isolated from leaf tissues (Fig. 3). A 540 bp DNA fragment, including both 5' and 3' flanking sequences of the CTB:SEKDEL fusion gene, was amplified from transformed potato genomic DNA. No nonspecific amplification was observed in any of the samples, indicating a high specificity of primers used for the PCR reaction. The DNA fragment amplified from plasmid vector pPCV701FM4-CTB:SEKDEL (Fig. 3, lane 2) and the DNA fragments amplified from transformed plant genomic DNA (lanes 4-6) migrated to the same position in the gel. Although, identical amounts of template genomic DNA (500 ng) was used for the PCR reaction, the plant exhibiting the highest luciferase activity (Fig. 2, plant No. 4) also demonstrated the highest level of PCR amplification (Fig. 3, lane 6). The correlation between PCR product level and luciferase activity suggests the possibility of increased T-DNA copy number in this transformant.

Immunoblot analysis of plant synthesized CTB protein

The three selected transformed plants were analyzed for the presence of CTB protein by immunoblot analysis (data not shown). Plant No. 4 which demonstrated the

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highest detectable CTB level was selected for CTB protein characterization. Transgenic potato leaf callus tissues were shown to contain CTB protein that strongly reacted with anti-cholera antibodies produced against the cholera holotoxin (Fig. 4A). The presence in bacterial CTB preparations of a band with an approximate molecular weight of 85 kDa (Fig. 4A, lanes 1 and 2, and B, lane 1) suggests the presence of cholera toxin A subunit (27 kDa) as part of the heterohexameric AB_5 holotoxin complex (Zhang *et al.*, 1995). Owing to its recombinant origin, plant-synthesized CTB did not show the presence of A subunit (Fig. 4A, lane 4). Homogenates from untransformed plants did not interfere with the antigenicity of exogenously added cholera toxin B subunit (Fig. 4A, lane 2). Anti-cholera toxin antibodies did not show a significant cross reaction with potato plant proteins (Fig. 4A, lane 3). Plant-produced CTB protein dissociated into monomers with molecular weight of approximately 15 kDa when the homogenate was boiled for 3 min prior to SDS-PAGE (Fig. 4B, lane 4). Comparative Coomassie blue staining of boiled and unboiled CTB proteins after SDS-PAGE showed that disappearance of multimeric CTB was due to disassembly of CTB oligomers rather than due to protein degradation (data not shown). Heat-induced dissociation of CTB multimers (Fig. 4A, lane 4, B lane 5) into monomers (Fig. 4B, lane 4), as previously demonstrated for bacterial enterotoxins (Gill, 1976; Hirst and Holmgren, 1987; Hardy *et al.*, 1988), confirmed the oligomeric (possibly pentameric) status of CTB protein in transformed potato tissues. Acid-induced dissociation of plant-derived CTB protein, similar to that seen for bacterial enterotoxin (Hardy *et al.*, 1988), was also detected (data not shown). However, dissociation into the monomeric form was not as complete as observed after heat treatment. These results suggest that the plant ER, similar to the periplasmic space of Gram-negative bacteria (Hirst and Holmgren, 1987), provides an intracellular environment in which monomeric B subunits are concentrated and assembled into oligomeric form. Presence of the SEKDEL hexapeptide at the C-terminus of proteins has been shown to significantly increase protein accumulation within plant tissues such as potato leaves and tubers, tobacco and alfalfa leaves as well as in COS cells (Munro and Pelham, 1987; Wandelt *et al.*, 1992; Haq *et al.*, 1995), thereby facilitating protein subunit oligomerization. Immunoblot analysis of plant-synthesized CTB showed that the monomers were significantly less immunoreactive in comparison to the multimeric form (Fig. 4B,

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compare lanes 4 and 5). Similarly, bacterial CTB was found to be substantially less reactive when dissociated by heat. The use of antibody raised in rabbits against cholera holotoxin, which may recognize native conformation of CTB pentamer molecules more efficiently than denatured and altered subunit conformation of CTB monomers, may explain this result. Pentameric CTB protein progressively dissociated into the monomeric form upon storage at 4 °C over a period of several months (Fig. 4B, lane 1). Both multimeric and monomeric forms of plant-derived CTB were of slightly higher molecular weight than bacteria-derived CTB (50 kDa versus 45 kDa for the pentamer and 15 kDa versus 12 kDa for the monomer, respectively). This result may be due to presence of the hexapeptide ER retention signal, or possibly to failure of plant cells to remove the leader peptide. Molecular weight differences of approximately 3 kDa between the monomers of bacteria and plant-derived CTB (Fig. 4B, lanes 1 and 4) suggest that the presence of both ER retention signal and extra N-terminal peptide contribute to the molecular weight increase of the plant-synthesized CTB molecule. Based on gel electrophoresis data, the apparent molecular weight of bacterial and plant CTB multimers is slightly smaller than the molecular weight of their monomers multiplied fivefold, which suggests a compact oligomeric configuration.

Transgenic potato microtuber tissues were analyzed for the presence of multimeric CTB proteins (Fig. 4C). Following auxin induction, homogenates prepared from microtuber tissues revealed biochemical characteristics of multimeric CTB protein identical to that found in leaf callus tissues, confirming auxin-induced CTB gene expression in all tissues of transformed potato plants.

CTB protein levels in transformed potato plants

To obtain quantitative estimates of CTB protein levels in leaf callus tissues of transgenic plant No. 4, both chemiluminescent ELISA and chemiluminescent immunoblot assays were employed.

In the chemiluminescent ELISA method, the amount of plant CTB protein was measured by comparison of the relative light units (RLU) from a known amount of bacterial CTB protein-antibody complex with that emitted from a known amount of

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transformed plant soluble protein. The amount of CTB detected was expressed as a percentage of total soluble plant protein (% CTB) in the sample (Fig. 5). Optimal concentrations of soluble protein loaded in the wells of the microtiter plate yielded CTB protein levels reaching 0.3% of total soluble protein in auxin-induced potato tissues (1.5 ng of plant CTB protein detected in 0.5 μg of total plant soluble protein). When the concentration of total protein deviated from optimal levels, the amount of CTB protein detected decreased. This result may be due to the binding characteristics of the microtiter plate wells to CTB protein in a mixture of total plant proteins. With increasing plant protein levels, increasing amounts of CTB protein may be unable to bind to the wells and is eventually lost through washing. Alternatively, the sensitivity of CTB detection may decrease with lower plant protein amount, eventually reaching undetectable levels.

In the chemiluminescent immunoblot method, luminescent intensities of bacterial and plant CTB protein bands blotted on Immun-Lite membranes after SDS-PAGE were measured by the Argus-100 low-light imager Data Analysis Program. The number of photons emitted from either bacterial CTB (Fig. 4B, lane 1) or plant CTB (Fig. 4B, lane 5) protein bands was quantified, and their values compared to provide a semi-quantitative estimate of the amount of plant synthesized-CTB protein. Based on the amount of light emission detected from a known amount of bacterial CTB protein (100 ng), the amount of plant CTB protein was calculated to be approximately 350 ng. The percentage of CTB protein in the plant was calculated based on the amount of soluble plant protein (100 μg) used in the assay. Based on this method, the percentage of plant CTB protein was found to be approximately 0.35% of total soluble plant protein, a value in close agreement with measurements made by the chemiluminescent ELISA method.

Based on the results of the chemiluminescent ELISA and immunoblot assays, 1 g of callus tissues (fresh weight) obtained from auxin-induced potato leaves contained 30-35 μg of recombinant plant CTB protein. In a parallel set of experiments, auxin-induced microtuber tissues were analyzed for the expression of CTB protein. The potato tuber tissues produced approximately 2-3 fold less CTB protein than detected in leaf callus tissues (compare Fig. 4A lane 4 with Fig. 4C lane 5).

Transgenic plant No. 4, with the greatest PCR amplification of the CTB fusion gene (Fig. 3, lane 6 versus lanes 4 and 5), exhibited the highest luciferase activity (Fig. 2,

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row 4 AB versus rows 1-3 AB and 5-6 AB) and the highest CTB protein accumulation, indicating that increased T-DNA copy numbers in the plant genome can contribute to increased expression levels of genes driven by the *mas* P1 and P2 promoters. In addition to increased copy numbers of T-DNA in the plant genome, favorable random position effects of the incoming gene may also explain range of protein levels between individual transformants.

Binding capacity of plant-derived CTB protein for the G_{M1} receptor

In G_{M1}-ELISA binding assays, plant-produced CTB protein, as well as bacterial CTB and CTX proteins, demonstrated a strong affinity for G_{M1}-ganglioside but not for BSA, or alternative sugar molecules, such as sucrose and galactose (Fig. 6). The ability of plant-derived CTB to bind G_{M1}-ganglioside indicates that the specific protein-ganglioside binding interactions between amino acid residues forming the G_{M1} binding sites and the oligosaccharide moiety of G_{M1}-ganglioside are conserved. Unlike the heat-labile enterotoxin of enterotoxigenic *E. coli*, cholera toxin does not bind to G_{M2}-ganglioside, which lacks the terminal galactose found in G_{M1} (Fukuta *et al.*, 1988). However, plant-derived CTB, bacterial CTB and CTX showed a weak but significant binding affinity for G_{M2}-monosialoganglioside (possibly due to G_{M1} contamination of the commercial G_{M2} preparation). Based on DNA sequence analysis of the CTB gene present in our plant expression vector, plant-synthesized CTB should contain tryptophan at position 88 of the protein. Tryptophan at this location is essential for both pentamerization of the B monomer as well as binding of the pentamer to the oligosaccharide moiety of G_{M1} (de Wolf *et al.*, 1981a,b). Boiling the plant and bacterial CTB prior to G_{M1}-ELISA abolished anti-CTB antibody detection (result not shown). This result could be explained by the fact that monomeric CTB is unable to bind to G_{M1}-ganglioside, and/or that monomers are weaker in antigenicity in comparison with oligomeric CTB. The strong binding efficiencies of plant and bacterial CTB for G_{M1} indicate that plant-derived CTB subunit binding to G_{M1} is cooperative (Schön and Freire, 1989; Merritt *et al.*, 1994). The apparent cooperativity exhibited by plant CTB-G_{M1}-ganglioside binding reinforces the probability that monomeric B subunits accumulate

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within the lumen of the ER of plant cells where self-assembly into oligomeric, possibly pentameric, G_{M1} binding forms occur.

The mas promoter system for expression of multiple genes

Auxin induction of the *mas* P1 and P2 promoters (Langridge *et al.*, 1989) results in expression of foreign proteins in plants at levels equivalent to or greater than strong constitutive promoters such as the cauliflower mosaic virus (CaMV) 35S promoter (Mason *et al.*, 1992; Haq *et al.*, 1995; Mason *et al.*, 1996). Auxin induction provides a substantial contribution to CTB gene expression. Approximately 100-fold lower amounts of CTB protein and luciferase activity were detected in leaf and tuber tissues without induction by this plant hormone (results not shown). While in potato transformation experiments, exogenous auxin addition was required to stimulate CTB gene expression from the *mas* promoter in leaf and microtuber tissues, food plants like tomato which make large amounts of auxin during fruit ripening may not require auxin induction for maximum gene induction.

The combination of a convenient method for screening large numbers of transformants via the bacterial luciferase reporter gene and the hormone-inducible *mas* promoter system provides the potential for rapid screening of large numbers of transgenic plants to select those with the highest transgene expression levels. In the potato transformation experiments, the luciferase reporter gene was not essential for identification of transformants as only six kanamycin-resistant plants were regenerated and all of them were observed to contain the CTB gene sequence. However, when plant transformation efficiency is low, presence of the luciferase gene on the *mas* P1 promoter can be extremely useful for selection of transformed plants. The results of our experiment indicate that the *mas* dual promoter system can serve as a model system for simultaneous expression of two desired gene products in food plant tissues. The *mas* dual promoter system provides a distinct advantage when two proteins are expected to be produced in the plant, for example, when two recombinant protein antigens are desired for construction of a vaccine against multiple pathogens.

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Future perspectives for food plant-derived recombinant CTB

The production of oligomeric CTB protein in edible plants may induce mucosal and systemic anti-cholera toxin antibodies in mammals at levels sufficient to provide protective immunity against cholera toxin challenge upon feeding transgenic plant tissues. Although, it has been reported that a production of enterotoxigenic *Escherichia coli* heat-labile enterotoxin B subunit (LT-B) in transgenic potato tubers of 0.01% of total soluble protein is sufficient to elicit production of both systemic and mucosal antibodies in mice with *in vitro* toxin neutralization capabilities upon oral immunization (Haq *et al.*, 1995), it may be desirable to increase antigen expression levels to create more effective food plant-based oral vaccines for larger animals and humans. Earlier reports indicate that cost-effective plant-based oral vaccines for developing countries will require increased amounts of recombinant proteins made in plants before they can be used as economically feasible production and delivery systems capable of replacing traditional bacterial fermentation systems (Mason and Arntzen, 1995). Minimum levels of recombinant protein antigen must be determined in food plants for each vaccine target, as individual protein antigens may vary in their ability to stimulate the immune response. In addition, palatable food plant species of tropical and semi-tropical origin that can be consumed without cooking, such as bananas, tomatoes and avocados, must be evaluated for their abilities to produce vaccine antigens. These food plants would be more practical alternatives for production of heat-labile recombinant protein antigens like LT-B or CTB, and due to their palatability, they would be particularly useful as oral vaccines for children. Development of edible transgenic plants with high expression levels of multimeric CTB, which can function both as an immunogen as well as a carrier peptide for other antigen epitopes, will ultimately provide a low-cost, convenient, effective, and safe strategy for the prevention of infectious and autoimmune diseases in man, especially in regions of the developing world where the resources of modern medical technology are largely unavailable.

Transgenic potato plants producing pentameric CTB.

The oligonucleotide sequence encoding the endoplasmic reticulum (ER) retention signal (SEKDEL) was fused to the 3' end of the CTB gene and cloned into the plant

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expression vector pPCV701FM4 (Fig. 7A). Following *Agrobacterium*-mediated potato leaf transformation, the CTB fusion gene was expressed in both microtuber and leaf tissues up to 0.3% of total soluble protein. In addition, the chimeric protein monomers assembled into pentamers which exhibited native antigenicity with a small molecular weight increase in comparison with the bacterial CTB pentamer (45 kDa vs. 50 kDa). The increase in molecular weight is presumably due to the extra six amino acids present at the C-terminus and possible retention of the 21-amino acid leader peptide at the N-terminus (Fig. 7B, compare lanes 1 and 3). This chimeric CTB molecule bound specifically to G_{M1}-ganglioside and thus retained the native biological activity of CTB. Both ELISA and low-light image analysis methods indicated that 1 g of transgenic potato microtuber or leaf callus tissue produced approximately 30 μg of pentameric CTB, and that the amount of CTB was uniform in the transformed potato tissues. Humans, unlike mice, eat only cooked potatoes, therefore the effect of boiling on pentameric CTB levels in potato tuber tissues was studied. After cooking the transgenic potato tubers in boiling water until the tissue became soft, approximately 50% of CTB was detected as the pentameric G_{M1}-ganglioside binding form (Fig. 7C).

Induction of serum and mucosal anti-CTB antibodies.

Potato tissues were orally administered to mice four times at weekly intervals for a month with a final booster feeding. Systemic and mucosal CTB-specific antibody titers were determined in both serum and feces collected from immunized mice by the class-specific chemiluminescent ELISA (CL-ELISA) method and expressed as endpoint (E) titers (Jackson, R. *et al.*, 1996) Essentially, the E titers for the three antibody isotypes (IgM, IgG, and IgA) were determined in serum and fecal samples as shown in Fig. 8A. Following subtraction of the background signal from mice fed untransformed potato tissues, relative light units (RLU) were plotted in log scale against two-fold dilutions (log_2) of serum or fecal samples to generate a titration curve. The curves were extrapolated for the three groups of mice to the level of 0.5 RLU, which was the value of the signal generated from the enzyme-substrate reaction alone in this assay system. Since the baseline RLU value determines the E titer, any fluctuation in RLU baseline affects the E titer value. Thus, errors in E titer estimations were calculated based on the deviation of

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RLU baseline values from 0.5 RLU, which was found to be in the range of 0.3 to 0.7. Regression coefficients are indicated for all three samples.

The E titers of the three serum anti-CTB antibody isotypes (IgG, IgA and IgM) were determined and the results expressed for days 35 and 70 (Fig. 8B, lanes 1–3). Anti-CTB titers for IgG and IgA slightly increased from day 35 to day 70, while the IgM titer decreased. The E titers of fecal (intestinal) anti-CTB antibody isotypes (IgA and IgG) were determined in a similar fashion, and the results expressed for each day of sample collection (Fig. 8C). Both fecal IgA and IgG titers reached the highest level around day 28, four days after the 4th feeding, and gradually decreased over the next 40 days until the booster feeding on day 65. In all three groups of CTB immunized mice, fecal IgA and IgG titers increased after the booster dose. For mice fed 3 g of transformed potato, a single booster feeding increased the IgA titer to approximately the highest titer observed on day 28. In general, fecal IgA titers were higher than IgG titers for three groups of immunized mice for each day of fecal sample collection. Mice immunized with 30 μg of bacterial CTB and 3 g of transformed potato tissues showed similar mucosal IgA antibody titers on day 70 of the ileal loop ligation experiment.

Neutralization of CT by anti-CTB antiserum.

Normal and affected Vero cell morphology was detected by light microscopy. Vero cells affected by cholera toxin appeared refractile, thick-walled, and possessed filamentous tendrils. The neutralization titer was defined as the highest serum dilution providing complete neutralization of CT cytotoxicity. Serum derived from mice fed 1g of untransformed potato tissues showed no protection against CT-induced cytotoxicity, and addition of immune serum alone to the cell monolayers did not adversely affect cell morphology, indicating that mouse serum contains no factors which abrogate the toxin effect, nor factors which alter Vero cell morphology. Mice orally immunized four times followed with a final booster of 30 μg bacterial CTB showed a titer of 1:32 for complete protection, in comparison with a titer of 1:8 for mice fed with 3 g of transformed potato tissues and a titer of 1:2 for mice fed with 1g of transformed potato tissues (Table 1). In these studies, cholera toxin neutralization titers are expressed as the highest dilutions of

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100 μL of pooled immune serum which conferred complete protection against cholera toxin (25 ng/mL final) cytotoxic effects in Vero cells.

Table 1. Neutralization of CT activity by immune serum

Serum samples	Titer
Mice gavaged	
Bacterial CTB (30 μg)	32
Mice fed transformed	
Potato tissue (1g)	2
Mice fed transformed	
Potato tissue (3g)	8

Reduction of CT-induced diarrhea in immunized mice.

Representative ileal loops from a mouse fed untransformed potato tissues and a plant-CTB immunized mouse are shown in Figs. 9A and B, respectively. To evaluate the protective efficacy of oral immunization, the volume of fluid accumulated in ileal loops from immunized and non-immunized animals was measured and expressed as the ratio of volume (V) to loop length (L) [V/L (μL/cm)] (Fig. 9C). Mice orally immunized with bacterial or plant-derived CTB showed significantly less fluid accumulation in comparison with mice fed untransformed potato tissues. Mean values of V/L for each group of mice were: 157.1 ± 12.5 SEM (standard error of the mean) for untransformed potato immunized mice; 71.0 ± 7.7 SEM for 30 μg bacterial CTB immunized mice; 91.0 ± 8.6 SEM for 1 g transgenic potato fed mice; and 59.9 ± 10.0 SEM for 3 g transgenic potato fed mice. Student's *t*-test revealed significant fluid reduction between the unimmunized mouse groups and the bacterial CTB-immunized mice ($P < 0.05$), and the transgenic potato-immunized mice [both 1 g and 3 g fed mice ($P < 0.05$)]. Considering reduction in intestinal fluid accumulation as a measure of protective efficacy of oral immunization, approximately 55% protection was observed for mice immunized with 30 μg of bacterial CTB, 42% protection for mice immunized with 1 g of transformed potato

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tissues, and 62% protection for mice immunized with 3 g of transgenic potato tissues. Ileal loops injected with saline did not accumulate significant amounts of fluid (1.5–3.5 μL/cm), suggesting that mechanical disturbances during loop ligation do not cause an inflammatory response.

Antiserum-mediated inhibition of cholera toxin - G_{M1}-ganglioside binding.

Plant-synthesized CTB was shown to specifically bind to G_{M1}-ganglioside, but not to mono- or di-saccharides such as galactose and sucrose. A G_{M1}-ELISA was performed to determine if CT neutralization in Vero cell cultures and mouse ileal loops was due to anti-CTB antibody-mediated prevention of CT binding to G_{M1}-ganglioside. Incubation of CT with pooled antiserum from mice fed 1 g of transgenic potato tissues resulted in 36% less RLU signal in comparison with the same amount of CT incubated with pooled antiserum derived from mice fed 1 g of untransformed potato tissues (Fig. 10, GM1+). However, when CT was incubated with either immune or non-immune serum, and the mixture bound to the microtiter plate surface not coated with G_{M1}-ganglioside, similar RLU signal levels were detected for both mixtures (Fig. 10, GM1–). The data represent mean values of RLU ± SD of four multiple samples.

The 21-amino acid leader peptide of the CTB protein, absent from secreted *V. cholerae* CT molecules, is thought to function as a leader peptide in eukaryotic cells for translocation of nascent CTB polypeptides into the lumen of the plant ER (van Heijne, G., 1985). The ER retention signal (SEKDEL) fused to the C-terminus of the plant CTB may sequester the protein within the plant ER and may increase stability of the protein (Haq, T., *et al.*, 1995; Wandelt, C., *et al.* 1992). The plant ER may function similarly to the periplasmic space of gram-negative bacteria in providing an intracellular environment in which CTB monomers accumulate and assemble into pentamers. It was previously demonstrated that the disassembled plant-synthesized CTB monomer was approximately 3 kDa larger than the bacterial CTB monomer (15 kDa vs. 12 kDa), which may be due to the presence of the hexapeptide ER retention signal and (or) the 21-amino acid leader peptide on the CTB molecule.

Many infectious agents which invade through mucosal surfaces can be effectively controlled by parenteral immunization, however, oral immunization is more effective for

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certain enteric diseases, resulting from *V. cholerae* and salmonellae infections. In the present study, oral immunization of CD-1 mice with pentameric CTB synthesized in potato plants induced generation of both mucosal and systemic anti-CT antibodies at levels sufficient to generate protective immunity against the biological effects of CT. The extent of cholera toxin neutralization in both Vero cell and ileal loop experiments suggests that anti-CTB antibodies prevent CT binding to cellular G_{M1}-ganglioside. The reduction in RLU signal detected when CT is mixed with immune serum in comparison with mixtures of CT and nonimmune serum (Fig. 10, GM1+), is unlikely to result from competition between mouse anti-CTB antibody and primary rabbit anti-CTB antibody for binding sites on the CT molecule, as there was no significant difference in RLU signal levels when CT was mixed with either immune or nonimmune serum prior to addition to microtiter plate wells not coated with G_{M1}-ganglioside (Fig. 10, GM1−). Thus, immunological protection against CT may be due predominantly to anti-CTB antibody-mediated specific prevention of CT binding to cellular G_{M1}-ganglioside. The molecular mechanism responsible for inhibition of CT binding to G_{M1}-ganglioside may rely on conformational changes induced in the B subunit of cholera holotoxin by CTB-specific antibodies. These results are consistent with *in vivo* experiments in which anti-CT antibodies prevented CT from binding to the cell surface (Apter, F., *et al.* 1993).

Secretory IgA is more abundant than IgG in the intestinal lumen (~ 10:1), however, it was observed that in fecal samples, differences in IgA and IgG titers were smaller than might have been expected (Fig. 8C). This result may have been dependent on the 10-fold reduction in protease activity detected in fecal extracts in comparison with intestinal secretions (de Vos and Dick, 1991). The relatively protease free environment in feces may protect the IgG antibodies from proteolytic degradation. Secretory IgA is more stable than IgG against proteolytic attack due to the presence of the secretory component gained during transcytosis.

Reduction in fluid secretion into intestinal loops of plant-CTB immunized mice clearly indicated that plant-based oral immunization was as effective as oral immunization with bacterial CTB for protection of the intestine against cholera toxin. Mice fed 3 g of transgenic potato tissues and mice gavaged with 30 μg of bacterial CTB exhibited similar intestinal protection levels, but mice fed 1 g potato showed slightly

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lower levels of protection (Fig. 9C). One gram of potato tissues was estimated to deliver about 30 μg of CTB, which is equivalent to the bacterial CTB dose, thus these two groups of mice were expected to show similar antibody titers and intestinal protection levels. However, mice fed 1 g of potato tissue exhibited detectably lower systemic and mucosal antibody titers, Vero cell neutralization titers and intestinal protection from cholera toxin in comparison with mice gavaged with 30 μg of bacterial CTB. Thus, it may be that plant-delivered CTB is less effective in immune stimulation than the same amount of bacterial CTB, which could be due to differences in antigen delivery i.e., gavage versus chewing and prolonged digestion of plant tissues consumed intermittently over a several hour period. Further, plant tissues may contain factors which interfere with antigen presentation to gut-associated lymphoid tissue (GALT) (Haq, T., *et al.*, 1995). In addition, the sodium bicarbonate buffer used for bacterial CTB gavage to neutralize stomach acid might have contributed to reduction in CTB pentamer disassembly, or possibly the presence of trace amounts of holotoxin in commercial CTB preparations may have enhanced immune response. An additional explanation for the differences in immunogenicity in mice, without apparent differences in antigenicity *in vitro* between bacterial CTB and plant CTB, may be the differences in amino acid sequences between the two CTB forms, especially presence of the hexapeptide sequestration sequence at the C-terminus and possible retention of the 21-amino acid leader peptide at the N-terminus of the plant CTB molecule. It was reported earlier that amino acid addition at the N-terminus of the CTB molecule resulted in minor changes in the net conformation, which resulted in a decrease in G_{M1}-ganglioside affinity and oral immunogenicity (Dertzbaugh and Elson, 1993). Although 3 g of potato and 30 μg of bacterial CTB showed more than 10% difference in protective efficacy (62% vs. 55%), the error bars overlap substantially, suggesting that differences between the two treatments are not significant (Fig. 9C).

An apparent discrepancy was observed between serum and mucosal anti-cholera toxin titers and toxin neutralizing assays in the intestine and in cell culture. Based on serum antitoxin titers, one might expect no dose-response relationship would be observed between 1 g and 3 g fed mice in the cell protection assay. However, mice fed 3 g of potato exhibited a higher toxin neutralization titer than mice fed 1 g of potato (Fig. 8B and Table 1). Similarly, based on mucosal antitoxin titers, bacterial CTB was expected to

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provide the highest levels of intestinal protection. However, mice fed 3 g of potato showed a slightly higher level of intestinal protection than mice given bacterial CTB (Figs. 8C and 9C). These apparent internal inconsistencies between antibody titers and protection against enterotoxin suggest that antibody titer is not always the most reliable marker for protective efficacy, possibly because not all antibodies are equally neutralizing (Apter, F., *et al.*, 1993). An apparent dissociation between antibody titers and vaccine efficacy was observed both in mouse models as well as clinical vaccine trials in the field (Haq, T., *et al.*, 1995; Lycke, N., *et al.*, 1983; Svennerholm, A., *et al.*, 1984; Jertborn, M., *et al.*, 1986; Lycke, N., *et al.*, 1989; Clemens, J., *et al.*, 1991). Therefore, the present observations, combined with those of other investigators, imply that antibody titers may not always be the best predictable markers for protection against *V. cholerae* and its toxin, which emphasize the importance of efficacy studies for *in vivo* protection. The protection referred to here should be emphasized as being for protection of tissues (i.e., intestine) of immunized animals rather than protection of the animal itself from a lethal dose of cholera toxin, which could also be a useful alternative food plant-based vaccine efficacy study in the mouse model (Dragunsky, E., *et al.*, 1992).

The increased protective efficacy of a killed-whole cell vaccine strain mixed with CTB (BS-WC) in comparison with the killed-cell vaccine strain alone (WC) in a large-scale field trial in Bangladesh clearly demonstrated the significant protective immunogenicity of the CTB subunit (Clemens, J., *et al.*, 1991; Clemens, J., *et al.*, 1992). It should be emphasized, however, that induction of antitoxic immunity alone does not provide protection against pathological effects caused by the live *V. cholerae*. The antibacterial (vibriocidal, i.e., anti-lipopolysaccharide and anti-outer membrane protein antigens) immune mechanism is critical for prevention of *V. cholerae* infection (Levine, M., *et al.*,1983; Levine, M., *et al.*, 1979). Thus, it will be desirable in the future to produce edible vaccine plants containing, in addition to enterotoxin antigens, neutralizing epitopes of structural antigens such as pilus-associated adhesins for induction of antibacterial immunity (Langermann, S., *et al.*, 1997).

While CTB is not a strong adjuvant for co-administered antigens, due to its ability to bind to G_{M1}-ganglioside on the surface of mammalian intestinal epithelial cells especially M cells of the GALT, it can function as an effective carrier for induction of an

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increased mucosal immune response to polypeptides to which CTB is chemically or genetically conjugated (Svennerholm, L., 1986; Czerkinsky, C., *et al.*, 1989; Dertzbaugh and Elson, 1993; Holmgren, J., *et al.*, 1994). Thus, production of pentameric CTB in food plants is not necessarily confined to development of an anti-diarrheal vaccine against cholera enterotoxin; CTB may be even more useful for providing safe and cost-effective mucosal immunization against other enteric pathogens which can be effectively controlled by recombinant subunit vaccines. Increased immunogen concentration at the mucosal lymphoid tissues may reduce the requirement for high levels of antigen biosynthesis in food plants. In addition, CTB has recently been shown to function as a carrier molecule for conjugated peptides for induction of immunological tolerance (Bergerot, I., *et al.*, 1997; Sun, J., *et al.*, 1996; Sun, J., *et al.*, 1994). Thus, food plant CTB-based oral vaccines may open the way for a novel food plant-based therapeutic approach for prevention of autoimmune diseases such as type I diabetes (Bergerot, I., *et al.*, 1997) and encephalomyelitis (Sun, J., *et al.*, 1996).

Potato is an excellent species for experimental study in the newly emerging field of edible plant-based oral vaccines, because potato tissues are relatively easily transformed and regenerated into plants. Tubers are relatively rich in proteins (over 4% soluble protein), and experimental animals (i.e., mice) readily consume raw tubers. Humans, however, favor cooked potatoes which may result in extensive destruction of vaccine antigens especially heat-labile proteins such as LTB and CTB. However, after cooking tuber tissues to the soft state, approximately 50% of the pentameric CTB remained intact as the G_{M1}-ganglioside binding pentamers (Fig. 7C). Therefore, while alternative food plant species, which grow in developing countries and which are edible without heat treatment such as banana and tomato, should be pursued for their ability to produce and deliver mucosal vaccines, due to their high protein content, potato plants may still retain a practical value for vaccine production for humans.

A novel result of these animal immunization experiments is the indication that antibody titers can be boosted by oral administration of additional vaccine food plant tissues when the protective titer declines. Thus, food plants grown in tropical and semi-tropical regions of the world can provide a continuous source of oral vaccine for the inevitable booster dose. Application of the CTB pentamer as an effective carrier for

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conjugated peptides in food plants will move us closer to achievement of a low-cost, convenient, effective, and safe strategy for prevention of infectious enteric diseases in animals and in man, especially in regions of the economically emerging world where conventional vaccines are unaffordable as well as unavailable.

The present invention has been described with reference to particular examples for purposes of clarity and understanding. It should be appreciated that certain improvements and modifications can be practiced within the scope of the appended claims and their equivalents.

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The pertinent portions of the following references are incorporated herein by reference.

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WHAT IS CLAIMED IS:

1. A DNA construct that encodes a fusion protein comprising a subunit of an enterotoxin and a signaling peptide.
2. The DNA construct of claim 1, wherein the subunit is the cholera toxin B (CTB) subunit.
3. The DNA construct of claim 1, wherein the signaling peptide is a microsomal retention signal.
4. The DNA construct of claim 3, wherein the signal comprises the amino acid sequence Ser-Glu-Lys-Asp-Glu-Leu.
5. The DNA construct of claim 1, wherein the signaling peptide is at the C-terminus of the fusion protein.
6. The DNA construct of claim 1, which is operably linked to a plant functional promoter.
7. The DNA construct of claim 6, wherein the plant functional promoter is selected from CaMV 35S, tomato E8, patatin, ubiquitin, mannopine synthase P1, mannopine synthase P2, rice actin 1, *A. tumefaciens* gene 5 promoter, *B. mori* cytoplasmic actin promoter, and tandem repeats thereof.
8. The DNA construct of claim 1, further comprising a selectable marker gene.
9. The DNA construct of claim 8, wherein the selectable marker gene codes for antibiotic resistance or a visualizable protein.
10. The DNA construct of claim 9, wherein the visualizable protein is a luciferase, green fluorescent protein, glucuronosidase or β-galactosidase.
11. The DNA construct of claim 1, which is flanked by right and left T-DNA border regions of *A. tumefaciens*.
12. The DNA construct of claim 1, further comprising a nucleotide sequence encoding an antigenic polypeptide between said subunit and the signaling peptide.
13. The DNA construct of claim 12, wherein the antigenic polypeptide is a mammalian, bacterial, viral, or fungal peptide sequence.
14. The DNA construct of claim 12, wherein the antigenic polypeptide can assemble as an antigenic complex or particle.

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15. The DNA construct of claim 12, wherein said subunit acts as a carrier for the antigenic polypeptide.

16. The DNA construct of claim 1, further comprising a nucleotide sequence encoding a leader sequence at the N-terminus of the fusion protein.

17. An expression vector comprising the DNA construct of claim 1 and an *E. coli* origin of replication.

18. The expression vector of claim 17, further comprising an *A. tumefaciens* origin of replication.

19. A strain of *E. coli* transfected with the expression vector of claim 17.

20. A strain of *A. tumefaciens* transfected with the expression vector of claim 18.

21. The strain of *A. tumefaciens* of claim 20, further comprising a helper Ti plasmid.

22. A transgenic plant cell transformed with the DNA construct of claim 1.

23. The plant cell of claim 22, wherein said DNA construct is integrated into the nuclear genome of the cell.

24. The plant cell of claim 22, wherein the plant is selected from the group consisting of potato, tomato, banana, soybean, pepper, tobacco, wheat, rye, rice, spinach, carrot, maize and corn.

25. A transgenic plant seed transformed with the DNA construct of claim 1.

26. The plant seed of claim 25, wherein the seed is of a plant selected from the group consisting of potato, tomato, banana, soybean, pepper, tobacco, wheat, rye, rice, spinach, carrot, maize and corn.

27. A transgenic plant transformed with the DNA construct of claim 1.

28. The transgenic plant of claim 27, wherein said enterotoxin subunit is CTB subunit.

29. The transgenic plant of claim 27, wherein the DNA construct is integrated into the nuclear genomes of cells of the plant.

30. The transgenic plant of claim 27, which is selected from the group consisting of potato, tomato, banana, soybean, pepper, tobacco, wheat, rye, rice, spinach, carrot, maize and corn.

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31. A method of transforming a plant cell with the DNA construct of claim 1 comprising contacting the plant cell with a strain of *A. tumefaciens* as in claim 21 under conditions effective to transfer and integrate the construct into the nuclear genome of the cell.

32. A method of producing a transgenic plant comprising transforming a plant cell as in claim 31 and regenerating the plant cell.

33. A method of transforming a plant cell with the DNA construct of claim 1 comprising subjecting the plant cell to microparticle bombardment with solid particles loaded with the DNA construct of claim 1 under conditions effective to transfer and integrate the construct into the nuclear genome of the cell.

34. A method of producing a transgenic plant comprising transforming a plant cell as in claim 33 and regenerating the plant cell.

35. A method of producing an immunogen in a plant comprising cultivating the transgenic plant of claim 27 under conditions effective to express said fusion protein.

36. A method of inducing immunity to an enterotoxin in a mammal or avian comprising causing the mammal to consume an immunizing effective amount of an edible part of the transgenic plant of claim 27.

37. The method of claim 36, wherein the enterotoxin is cholera.

38. A method of effecting or boosting immunity of a mammal or avian to a pathogen comprising:

causing the mammal to consume an adjuvant effective amount of an edible part of the transgenic plant of claim 27; and

subjecting the mammal to a live, dead, or attenuated amount of said pathogen, or antigenic portion thereof.

39. The method of claim 38, wherein said enterotoxin subunit is CTB subunit.

40. The method of claim 38, wherein said antigenic portion is provided by the antigenic polypeptide of claim 12.

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INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 98/21237

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C07K14/28 C12N15/62 A61K38/16 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HAQ T A ET AL: "ORAL IMMUNIZATION WITH A RECOMBINANT BACTERIAL ANTIGEN PRODUCED IN TRANSGENIC PLANTS" SCIENCE, vol. 268, no. 5211, 5 May 1995, pages 714-716, XP002024034 cited in the application	1,3-11, 16-27, 29-36,38
Y	see the whole document	2,12-15, 28,37, 39,40

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 1999	25/02/1999
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	**Authorized officer** Oderwald, H

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 98/21237

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	NASHAR T O ET AL: "CURRENT PROGRESS IN THE DEVELOPMENT OF THE B SUBUNITS OF CHOLERA TOXIN AND ESCHERICHIA COLI HEAT-LABILE ENTEROTOXIN AS CARRIERS FOR THE ORAL DELIVERY OF HETEROLOGOUS ANTIGENS AND EPITOPES" VACCINE, vol. 11, no. 2, 1993, pages 235-240, XP000645274 see the whole document	2,12-15, 28,37, 39,40
A	JEFFERSON R A ET AL: "GUS FUSIONS: BETA-GLUCURONIDASE AS A SENSITIVE AND VERSATILE GENE FUSION MARKER IN HIGHER PLANTS" EMBO JOURNAL, vol. 6, no. 13, 20 December 1987, pages 3901-3907, XP000654406 see the whole document	16-35
P,X	ARAKAWA T ET AL: "A plant-based cholera toxin B subunit-insulin fusion protein protects against the development of autoimmune diabetes." NATURE BIOTECHNOLOGY, JOURNAL CODE: CQ3. ISSN: 1087-0156, vol. 16, no. 10, 1 October 1998, pages 934-938, XP002093387 see the whole document	1-40

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

BNSDOCID: <WO_____9918225A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/21237

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 36-40 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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